

STUDY ON THE IMMOBILIZATION OF PAPAIN WITH A MACROPOROUS BEAD CARRIER OF COPOLYMER CONTAINING MONOMER UNITS OF *N*-AMINOETHYL ACRYLAMIDE AND VINYL ALCOHOL*

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Abstract A kind of macroporous bead carrier of copolymer containing monomer units of *N*-aminoethyl acrylamide and vinyl alcohol was synthesized, i.e. the MR-AA carrier. Papain was immobilized on the carrier using glutaraldehyde as the coupling agent. The enzymatic activity of the immobilized papain was compared with free papain using casein as a substrate, and the effects of glutaraldehyde concentration, pH, temperature, time and papain amount added on the activity recovery were also investigated. The results show that the MR-AA carrier contains reactive primary amine groups, hydrophilic amido links and hydroxyl groups, as well as macroporous structures based on its matrix (MR-AV matrix), furthermore, the activity recovery of papain in the immobilization could reach 48%~58%. In comparison with free papain, the resulting immobilized papain exhibits a remarkable thermostability and better reusability.

Keywords Immobilization, Papain, Proteolytic enzyme, Macroporous bead carrier

INTRODUCTION

A series of polymer materials have been extensively applied to immobilization of enzymes because they have various functional groups and can be easily modified chemically^[1], and it was found that the carrier with macroporous structures would be more suitable for enzyme immobilization^[2]. Papain (EC.3.4.22.2) is one of the proteolytic enzymes^[3], particularly it has been employed in the synthesis of peptides and oligo-amino acids^[4-7], as well as in the fields of food and medicine production^[8, 9]. As a good model, papain has a wide range of potential applications in enzyme engineering, so there are numerous reports related to the preparation of immobilized papain^[10-15]. In this work, a macroporous bead carrier containing both *N*-aminoethyl acrylamide and hydroxyl groups (MR-AA carrier) was successfully synthesized by the reaction of the macroporous acrylonitrile (AN)-vinyl acetate (VAc)-divinylbenzene (DVB) terpolymer matrix (MR-AV matrix) with excess ethylenediamine containing water. Papain was immobilized on the MR-AA carrier by coupling reaction of glutaraldehyde. Meanwhile, the enzymatic activity of the resulting immobilized papain has been studied according to the procedure reported previously^[12], using casein as a substrate. The factors affecting the activity of the immobilized papain, such as glutaraldehyde concentration, pH, temperature, time and papain amount added were also investigated by the activity assay of the immobilized and free papain. In addition, composition and properties of the MR-AA carrier and MR-AV matrix were characterized by means of FT-IR, elemental analysis and mercury porosimetry.

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EXPERIMENTAL

Materials and Instrumentation

Papain (EC.3.4.22.2), Sigma Co., 17 U/mg; glutaraldehyde, E.Merck Co., 25% concentration; tris(hydroxymethyl)aminomethane (Tris), L-cysteine and casein are biochemicals; trichloroacetic acid, EDTA, AN, VAc, DVB and ethylenediamine are A.R. reagents. Elemental analysis was carried out by using a Carlo Erba elemental analyzer model 1106. FT-IR spectrum was recorded on a Nicolet FT-170 SX spectrometer. The pore parameters were measured by a Carlo Erba 2000 mercury porosimeter. UV absorbance was measured by a Shimadzu UV-240 spectrophotometer.

Preparation of the MR-AV Matrix

The MR-AV matrix was synthesized by suspension polymerization of AN, VAc and DVB using a linear poly(vinyl acetate) as a porogenic agent, according to the method reported previously^[2]; here the crosslinking degree of the matrix was controlled at 7%.

Synthesis of the MR-AA Carrier

10 g MR-AV matrix was placed in a 100 mL three-necked flask fitted with stirrer, reflux condenser and thermometer, then 30 g ethylenediamine containing 5% water was added. The synthesis reaction was carried out at 105~110 °C for 10 h. The MR-AA carrier was obtained by filtering, washing with distilled water until ethylenediamine-free and drying under vacuum.

Immobilization of Papain

6 mL of 0.05 mol/L Tris-HCl buffer (including 0.05 mol/L L-cysteine and 0.02 mol/L EDTA, pH 8.0) and 0.5 g MR-AA carrier were placed in a 25 mL flask fitted with a magnetic stirrer and were equilibrated for 1 h with stirring at 25 °C. Then 2 mg papain and 0.1 mL of 25% glutaraldehyde were introduced into the flask successively. The immobilization was carried out at 25 °C for 8 h with stirring, the reaction solution was removed by filtering, the resulting immobilized papain was washed with distilled water and 0.05 mol/L Tris-HCl buffer. Finally, it was dried in a desiccator with anhydrous calcium chloride at 0~5 °C.

Assay for Papain Activity

The activity of free and immobilized papain was determined according to the published methods^[3, 12] using casein as a substrate. The determination was performed by the following procedures:

Free Papain Activity

0.2 mg/mL papain solution was prepared by dissolving papain in 0.05 mol/L Tris-HCl buffer. Then 0.7 mL of 0.05 mol/L Tris-HCl buffer (pH 8.0), 0.2 mL of papain activator consisting of 0.5 mol/L L-cysteine and 0.02 mol/L EDTA (pH 8.0), 0.1 mL of the papain solution and 1 mL of 1% casein solution (pH 8.0) were introduced into a 10 mL flask successively with magnetic stirring. When the enzymatic reaction was carried out at 37 °C for 10 min, 3 mL of 5% trichloroacetic acid solution was added to the flask, the reaction mixture was kept at 25 °C for 1 h, then it was filtered. The absorbance of the filtrate obtained was measured at 280 nm.

Immobilized Papain Activity

A given amount of immobilized papain and 1.6 mL of the above Tris-HCl buffer were introduced into a 10 mL flask fitted with a magnetic stirrer and equilibrated at 25 °C for 1 h, then 0.4 mL of the papain activator and 2 mL of casein solution were put into the flask. The enzymatic reaction was carried out at 37 °C for 10 min with stirring, the immobilized papain added was removed from the reaction solution at once, 3 mL of 5% trichloroacetic acid solution was added to 2 mL of the reaction solution without immobilized papain, the mixed solution was maintained at 25 °C for 1 h and filtered. The absorbance of the filtrate obtained was measured at 280 nm.

RESULTS AND DISCUSSION

Composition, Structure and Properties of the Matrix and Carrier

The MR-AV matrix is obtained as yellowish beads of 0.08~0.32 mm particle size. The elemental analysis indicates that its nitrogen content reaches 21%, that is to say, the polyacrylonitrile (PAN) content in this copolymer should be 79% (wt%). The pore parameters obtained by mercury porosimetry were 6.20 m²/g specific surface area, 0.39 cm³/g specific pore volume and 800 nm average pore radius, respectively. The corresponding FT-IR spectrum and pore parameters are basically similar to the results reported previously^[2]. Then the MR-AV matrix was changed into the MR-AA carrier by reaction with ethylenediamine containing 5% water. The IR spectrum of MR-AA (Fig. 1) has characteristic absorptions of the secondary amide group (1671, 1560, 1290, 711 and 571 cm⁻¹), hydroxyl group (δ O—H, 1246 cm⁻¹; ν C—O, 1106 cm⁻¹) and primary amino group (δ N—H, 1648 cm⁻¹; ν C—N, 1067 cm⁻¹; ν N—H peak overlapped with ν O—H peak at 3451 cm⁻¹). The IR results indicate that a chemical composition consisting of *N*-aminoethyl acrylamide and vinyl alcohol has been formed by the above reaction^[2, 16]. The resulting MR-AA carrier holds 63% of water regain and 0.63 g/mL of wet apparent density found by the methods reported previously^[17], however, the nitrogen content found in the MR-AA carrier is 17.5% and less than that of its matrix, this should be the result of the incomplete conversion of cyano groups due to an ortho-group effect of the polymeric reaction, and there also exists a few complete hydrolysis of the cyano group in the reaction. Because of the inclusion of reactive primary amino group, hydrophilic amido and hydroxyl groups, an apparent density which is suitable for column operation, as well as good pore parameters resembling the MR-AV matrix, the MR-AA carrier should be favourable for enzyme immobilization^[2, 12].

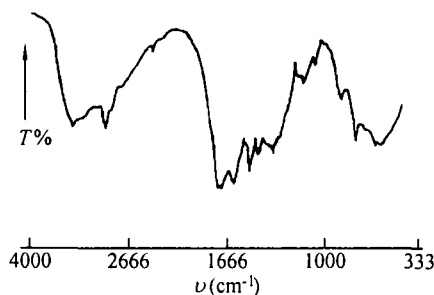


Fig. 1 FT-IR spectrum of MR-AA carrier

The Factors Affecting the Activity Recovery of Papain

Effect of glutaraldehyde concentration

Because glutaraldehyde is both a crosslinking agent and a denaturant for the immobilization of papain, the optimal glutaraldehyde concentration should be selected in order to obtain a higher activity recovery of papain. The highest activity recovery could be obtained by using 0.2% glutaraldehyde concentration as a crosslinking agent, and the activity recovery also reaches over 40% when glutaraldehyde concentration is changed from 0.1% to 1.5%, as shown in Fig. 2. This indicates that the denaturalization of glutaraldehyde to papain would be partially inhibited in the case of using the MR-AA carrier.

Effect of pH

The activity recovery obtained at various pH values is illustrated by Fig. 3. When the pH value of Tris-HCl buffer is at 7.5, the highest activity recovery could be obtained, while the activity recovery is at least 39% from pH 6.0 to pH 9.0. This shows that the suitability of papain to the medium acidity would be enhanced due to the MR-AA carrier.

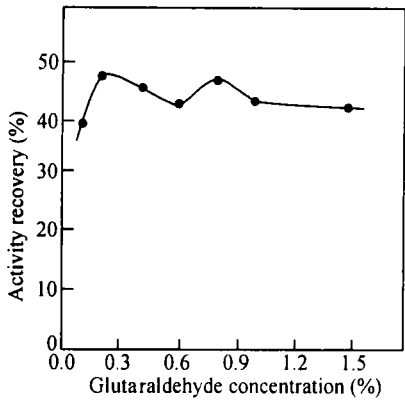


Fig. 2 Effect of glutaraldehyde concentration on the activity recovery

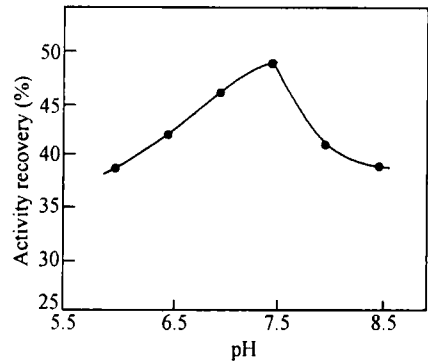


Fig. 3 Effect of pH value on the activity recovery

Effect of temperature

Figure 4 illustrates the effect of coupling temperature on the activity recovery. The highest activity recovery could be obtained at 30°C, and when the temperature was raised to 35, 40 and 45°C, the corresponding activity recovery could also reach 44%, 39% and 37% successively. This may be mainly due to the protection effect of macroporous structure of the MR-AA carrier on papain.

Effect of time

The highest activity recovery would be obtained after immobilization for 8 h, and the activity recovery would also reach 45% even if reaction time is 24 h, as shown in Fig. 5. However, a shorter coupling time will decrease of the activity loss.

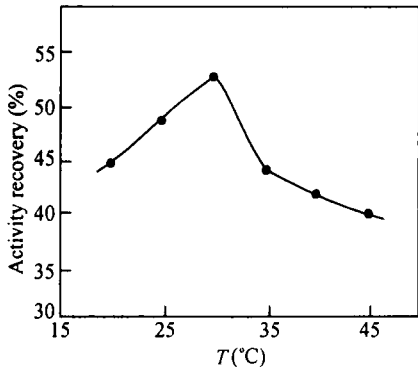


Fig. 4 Effect of immobilization temperature on the activity recovery

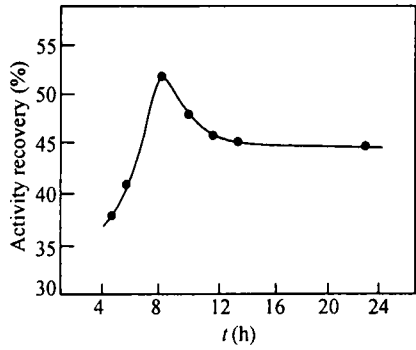


Fig. 5 Effect of immobilization time on the activity recovery

Effect of papain amount added

when the amount of carrier used was 50 mg, the effect on the activity recovery of different papain amounts added is illustrated in Fig. 6. Because a higher enzyme amount supported will make the enzyme to form an intermolecular space hindrance decreasing dispersion of the substrate and product^[18]. An addition of 0.25 mg papain could result in a higher activity recovery.

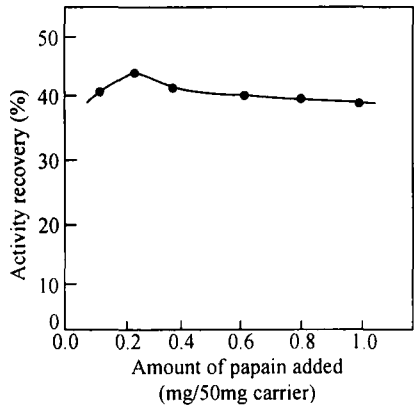


Fig. 6 Effect of the amount of enzyme added on the activity recovery

Properties of the Immobilized Papain

Effect of reaction temperature

The activity variation obtained when the immobilized and free papain were allowed to react with casein at given temperatures, is shown in Fig. 7. The optimal activity temperature of the resulting immobilized papain is 10°C higher than that of the free papain. Moreover, the immobilized papain still exhibits 69% of the residual activity at 80°C. These indicate that papain immobilized by the MR-AA carrier could extend its durability to a higher temperature.

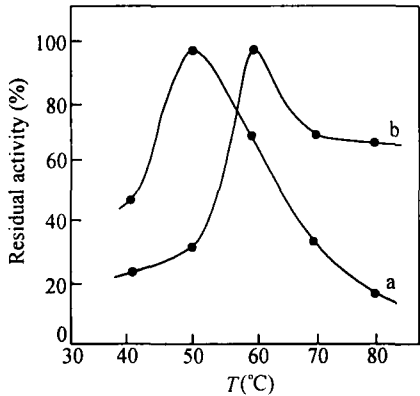


Fig. 7 Effect of reaction temperature on the enzymatic activity
a) free papain, b) immobilized papain

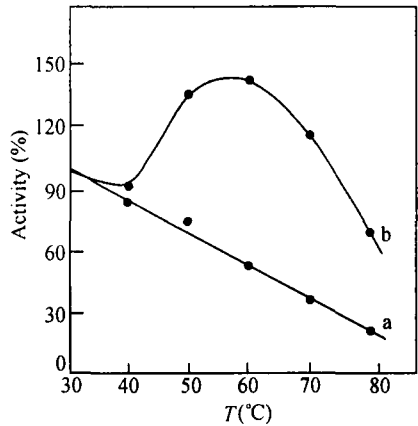


Fig. 8 Effect of storage temperature on enzymatic activity
a) free enzyme, b) immobilized enzyme

Thermal stability

After the immobilized and free papain suspended in 0.05 mol/L of Tris-HCl buffer without a substrate were stored for 1 h at the given temperature and cooled to 4°C again, the activity variations assayed by the above procedures are shown in Fig. 8. As the storage temperature was raised successively, the activity of free papain exhibited a basically linear decrease. However, the activity of the immobilized papain increased significantly from 50°C to 70°C. This is due to the thermal activation forming a optimal activity state for the immobilized enzymes, as described in previous papers^[2, 12, 15]. The resulting immobilized papain still holds 70% of its activity, while that of free papain is only 20% at 80°C. The results presented in Fig. 8 show that the papain immobilized by MR-AA carrier has good thermostability.

Effect of medium pH

Figure 9 illustrates that the activities of the immobilized and free papain vary with the pH of the reaction medium. Although both optimal pH values which give the highest activity are still at pH 7.0, the residual activity of the free papain decreases markedly from pH 8.0 to pH 10.0, while that of the immobilized papain is less affected, e.g., that of the latter is still 86% but that of the former has decreased to 28% at pH 10.0, as shown in Fig. 9. This further demonstrates that the resulting immobilized papain would exhibit a better stability for medium acidity.

Stability after repeated use

How the residual activity of the immobilized papain varies with reusing times is shown in Fig. 10. According to Fig. 10, the immobilized papain loses its activity very slowly. Specifically, it still retains 57% activity after being reused for ten times. This kind of good reusability should result from its remarkable stability.

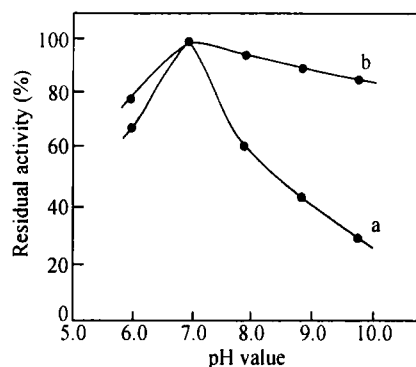


Fig. 9 Effect of medium pH on enzymatic activity
a) free enzyme, b) immobilized enzyme

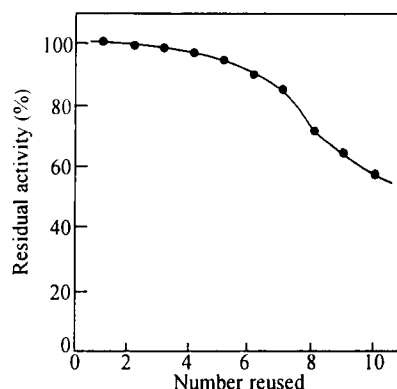


Fig. 10 Variation of the enzymatic activity with the number reused

CONCLUSIONS

The results of this research showed that papain could be well immobilized on the MR-AA carrier using glutaraldehyde as the coupling agent, and the resulting immobilized papain exhibits remarkable stability toward the medium pH, reaction and storage temperature, as well as a good reusability. The higher activity recovery obtained under given immobilization conditions further demonstrates that the MR-AA carrier would be very favourable for enzyme immobilization. Meanwhile, the MR-AA carrier could be easily prepared by the chemical modification of the macroporous bead matrix synthesized by suspension copolymerization of AN, VAc and DVB, using a linear poly(vinyl acetate) as a porogenic agent.

REFERENCES

- 1 Furusaki, S., J. Chem. Eng. Japan, 1988, 21: 219
- 2 Li, Y.F., Tao, G.L. and Zhuo, R.X., J. Mol. Catal.(China), 1992, 6: 440
- 3 Atkinson, B. and Mavituna, F., "Biochemical Engineering and Biotechnology Handbook", The Natural Press, Hongkong, 1985, p.508
- 4 Shin, C.G., Kakusho, T., Arai, K. and Seki, M., Bull. Chem. Soc. Japan, 1995, 68: 3549
- 5 Ulmann, D., Bordusa, F., Salchert, K. and Jakubke, H.D., Tetrahedron: Asymmetry, 1996, 7: 2047
- 6 Lozano, P., Avellaneda, A. and Iborra, T., Biocatal. Biotransform., 1996, 13: 255
- 7 Eberling, J., Braun, P., Kowalczyk, D., Schultz, M. and Kunz, H., J. Org. Chem., 1996, 61: 2638

- 8 Xin, G.W., Ye, Y.H. and Li, Z.X., *Chem. J. Chinese Universities*, 1998, 19: 1602
- 9 Groeger, U., Stehle, P., Furst, P., Leuchtenberger, W. and Drauz, K., *Food Biotechnol. (N.Y.)*, 1988, 2: 187
- 10 Muzzarelli, R.A.A., *Polysaccharides*, 1985, 3: 417
- 11 Sinisterra, J.V. and Alcantara, A.R., *J. Mol. Catal.*, 1993, 84: 327
- 12 Tao, G.L., Li, Y.F. and Zhuo, R.X., *Chinese J. Appl. Chem.*, 1993, 10: 9
- 13 Capllas, M., Serra, P., Benaiges, M.D., Caminal, G., Gloria, G. and Josep, L.S., *Biocatal.*, 1994, 11: 273
- 14 Bhardwaj, A., Lee, J., Glauner, K., Ganapathi, S., Bhattacharyya, D. and Butterfield, D.A., *J. Memb. Sci.*, 1996, 119: 241
- 15 He, F., Liu, L.J. and Zhuo, R.X., *Ion Exchange and Adsorption*, 1997, 13: 290
- 16 Li, Y.F. and Zhuo, R.X., *Chinese Chem. Lett.*, 1992, 3: 619
- 17 Zhou, C.H. and Li, Y.F., *J. Lanzhou University (Nat. Sci.)*, 1985, 21: 68
- 18 Lilly, M.D., Money, C., Hornby, W.E. and Merook, E., *Biochem. J.*, 1966, 98: 420